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Award Number: DAMD17-97-1-7157

TITLE: Breast Cancer Stimulation of Osteolysis

PRINCIPAL INVESTIGATOR: Merry J. Oursler, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Duluth, Minnesota 55812-2496

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (1 Sep 98 - 31 Aug 99)

4. TITLE AND SUBTITLE
Breast Cancer Stimulation of Osteolysis

5. FUNDING NUMBERS
DAMD17-97-1-7157

6. AUTHOR(S)
Merry J. Oursler, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Duluth, Minnesota 55812-2496

E-MAIL:
moursler@d.umn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

In this second year of funding, we have completed analysis of two tumors and have found mRNA and protein for GM-CSF, IGF-I and II, PTHrP, and TNF-α. We have also determined that TNF-α, but not GM-CSF, IGF-I or II, or PTHrP act as survival agents for osteoclast-like cells. When osteoclast-like cells differentiate in the presence of TNF-α, withdrawal of TNF-α once the mature cells are purified induces apoptosis while continued treatment with TNF-α represses apoptosis. We have also examined the activity of cells that are differentiated in the presence of PTHrP and TNF-α for resorption activity and lysosomal enzyme secretion. We have found that both treatments, either alone or in combination, result in more active osteoclast-like cells. All treatments resulted in increased resorption and secretion of cathepsin B, but only the cells that differentiated in the presence of TNF-α had elevated secretion of TRAP. We are continuing to examine the ramifications of these effects on activity and survival.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	44
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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PI - Signature 9/8/99 Date

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manuscript (submitted to Cancer Research and provisionally accepted):

Identification Of Breast Cancer Cell Line-Derived Paracrine Factors That Stimulate Osteoclast Activity. Authors: Larry Pederson, Bent Winding, Niels T. Foged, Thomas C. Spelsberg, and Merry Jo Oursler

INTRODUCTION

The purpose of this research is to examine granulocyte macrophage colony stimulating factor (GM-CSF), insulin-like growth factor II (IGF-II), tumor necrosis factor alpha (TNF-α), and parathyroid hormone related peptide (PTHrP) are present at the sites of breast cancer metastases in bone and determine the mechanisms by which breast cancer cells stimulate the activity of isolated osteoclasts *in vitro* by studying the effects of GM-CSF, IGF-II, TNF-α, and PTHrP on resorption activity, apoptosis, integrin expression, and secretion.

BODY

Overall, as detailed below, we have made excellent progress on this study. Many of the tasks are either completed and submitted for publication (manuscript appended) or are in the process of being completed. This report is detailed with specific reference to the Statement of Work.

Specific Aim 1: Determining if GM-CSF, IGF II, TNF-α, and PTHrP are present at the sites of tumor metastasis and likely to be involved in stimulating osteoclast activity.

Task 1: 1-12 months: PCR analysis of tumors.

This work was completed in year 1 and included in the previous progress report. The results of this aspect of the project are currently being incorporated into a manuscript.

Task 2: 5-16 months: Immunolocalization of factors in tumors.

We are still obtaining tumor samples for this aspect of the project. We have overcome some difficulties with developing sufficiently sensitive techniques that have low background staining. We anticipate conclusion of these studies in the final year of this project. Since we are behind on this aspect of the project, we have focused on Task 3 earlier than originally projected.

Task 3: 17-36 months: examine tumors for the presence of factors.

We have discovered that explants from surgical samples secrete IGF-I and II, GM-CSF, PTHrP, and TNF-α. The table below presents the results from two of our samples. We anticipate that these studies will be included with the publication of the PCR studies (Task 1).

	IGF-I (nM)	IGF-II (nM)	GM-CSF (nM)	TNF-α (nM)	PTHrP (nM)
tumor #1	1.93	0.84	0.10	0.60	10.05
tumor #2	2.05	2.19	0.29	0.89	14.11
tumor #2 CM	26.75	3.45	0.97	4.76	17.78

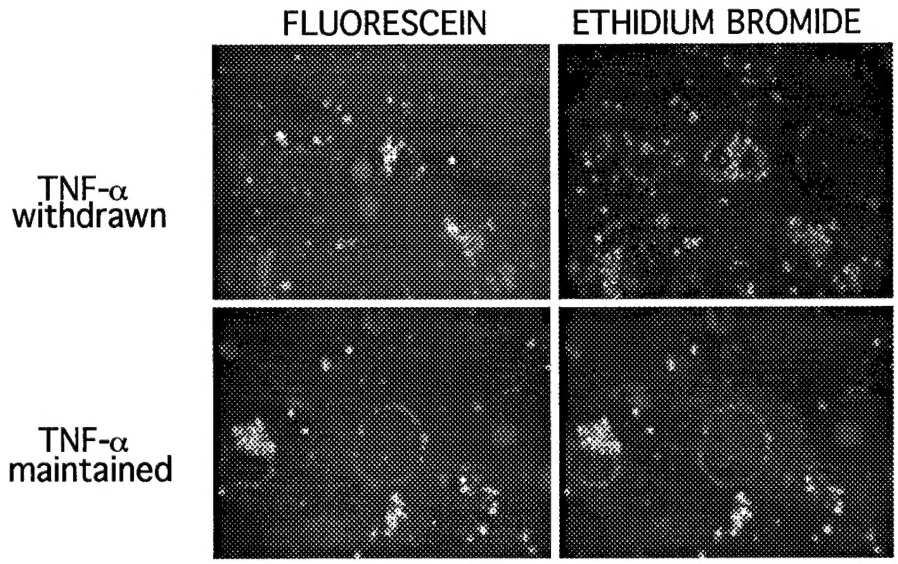
Osteolytic breast tumors from patients were analyzed by dot blotting. Dot blots were quantitated by densitometry using NIH Image 1.60. Values were compared to a standard curve and the quantity of each growth factor present in tumor tissue and conditioned media (CM) was calculated.

Specific Aim 2. Determine the mechanism by which breast cancer increase osteoclast resorption activity.

As detailed in our last progress report, we established a mammalian model system using *in vitro* generated mouse osteoclast-like cells (we had included IACUC approval notification in our last report). We have had concurrent studies taking place in both systems as each offered a separate strength: the avian system provided access to authentic osteoclasts and the mouse system produces mammalian osteoclast-like cells as well as having the additional benefit of allowing us to examine the influence that differentiation in the presence of the factor(s) on the phenotype of the mature cell. This has proven to be particularly important in our studies, as detailed below.

Task 4: 1-8 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on viability using the technique of fluorescein diacetate staining.

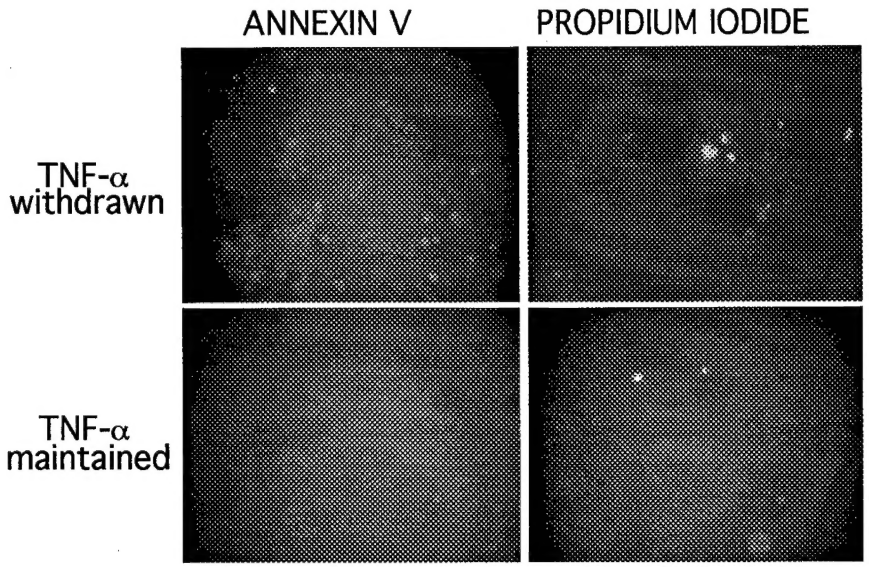
In both avian and mouse cells, we have examined GM-CSF, IGF-II, and PTHrP and have found no evidence that these influence osteoclast viability. Excitingly, we have discovered that TNF-α acts as a survival factor for mouse osteoclast-like cells that differentiate in the presence of 0.05 ng/ml TNF-α: We have examined the effects of withdrawal of TNF-α treatment once osteoclast-like cells differentiate and, as shown in the figure below, there are more dead cells (positive with ethidium bromide staining) in the withdrawn cultures compared to the TNF-α maintained cultures. This is being pursued as outlined in Task 5.



Analysis of cell survival using LIVE/DEAD fluorescent assays: A combination of permeant and impermeant fluorochromes are used to initially assess whether cells are living or dead, as defined by having a functional cell membrane. The LIVE/DEAD kit is used according to manufacturer's instructions to distinguish this category of cells (Molecular Probes). Briefly, cell cultures are incubated in a combination of calcein AM and ethidium homodimer-1, and examined with an epifluorescence microscope equipped with rhodamine and fluorescein filter sets. Esterase activity cleaves calcein AM which traps the green fluorescing calcein (on the left); dying and dead cells become permeable to ethidium homodimer-1 and fluoresce red (on the right). In the top panels, more cells are seen in the right panel than in the left. This indicates more dead than living cells. In the bottom panels, there are no cells positive in the right panel that are not positive in the left panel. This shows that there are fewer apoptotic cells compared with the top panel.

Task 5: 9-28 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on apoptosis with TUNEL analysis.

As noted above, we are focusing on TNF-α influences on osteoclast apoptosis in the mouse system. At this time, we are pursuing studies to examine the time frame of the survival effects of TNF-α as well as examining other aspects of apoptosis including membrane lipid changes (annexin V binding). As shown in the figure below, cells from which TNF-α is withdrawn are in early stages of apoptosis compared to cells maintained in 0.05 ng/ml TNF-α. Our observation of influences of TNF-α on survival are included in an abstract that will be presented at this year's American Society for Bone and Mineral Research meeting. We are currently using the TUNEL assay to further examine this observation.



Early Stage Annexin V staining: We are studying early stage of apoptosis by examining alterations in the phospholipid composition of the plasma membrane. Thus, detection of the externalization of phosphatidylserine is a mechanism by which membrane changes during apoptosis are detected. Annexin V binds to phosphatidylserine. Early stages of apoptosis are detected by the use of fluorescently tagged Annexin V to detect externalization of phosphatidylserine. FITC-conjugated Annexin V is used according to manufacturer's instructions (Oncogene Research Products), and membrane fluorescence compared between experimental and control groups. Propidium iodide staining is used to detect mid (cytoplasmic staining) and late (nuclear staining) apoptosis.

Task 6: 1-12 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on attachment.

This aspect of the project has been concluded in avian cells and is detailed in the accompanying manuscript. Specifically, we have observed that GM-CSF has no influence on attachment whereas IGF-II, PTHrP, and TNF-α each increase osteoclast attachment. Studies in the mouse system are ongoing.

Task 7: 5-28 months: Examine effects of GM-CSF, IGF II, TNF-α, and PTHrP on on integrin expression.

On the basis of the above studies, we are currently examining the effects of GM-CSF, IGF-II, TNF-α and PTHrP on osteoclast integrin expression in the final year of funding using the mouse

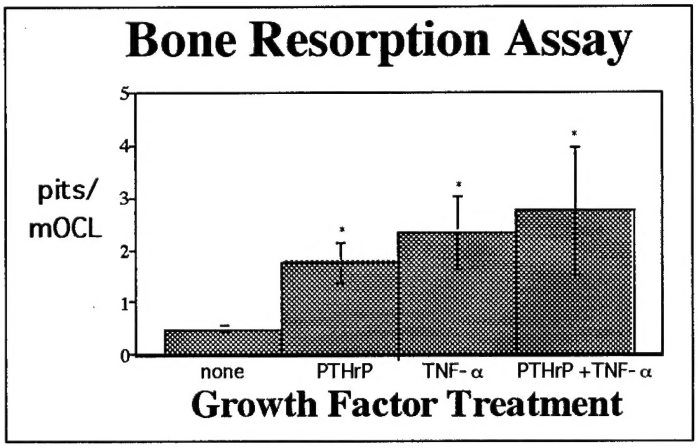
system. We have delayed the initiation of these studies until we completed the studies of attachment. Due to this delay, we have been able to progress quickly on Task 8.

Task 8: 17-36 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on lysosomal enzyme secretion.

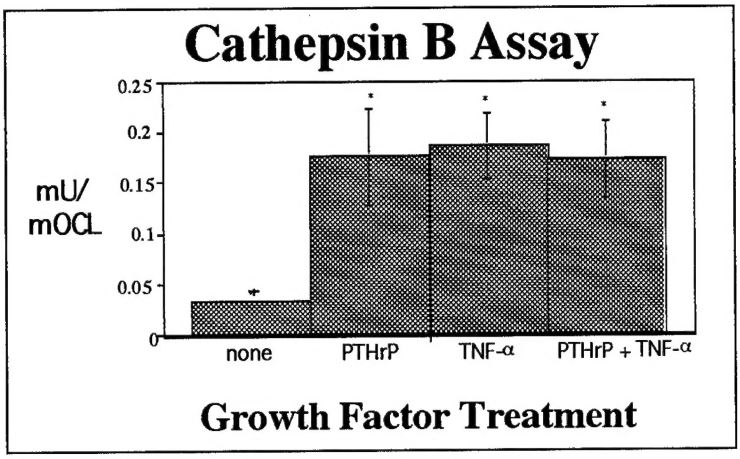
We have completed these studies using authentic avian osteoclasts and these studies are included in the appended manuscript. Specifically, we have determined the GM-CSF had no effect on lysosomal enzyme secretion. IGF-II, PTHrP, and TNF-α each stimulated secretion of cathepsin B and tartrate resistant acid phosphatase.

We are currently investigating the influence of differentiation in the presence of PTHrP, and TNF-α on mouse osteoclast-like cell bone resorption and lysosomal enzyme secretion (see figures below). We have evidence that these factors both individually and combined increase resorption and cathepsin B secretion. Interestingly, only TNF-α increases TRAP secretion. We are currently examining the effects and interactions between GM-CSF and IGF-I and II in the final year of funding.

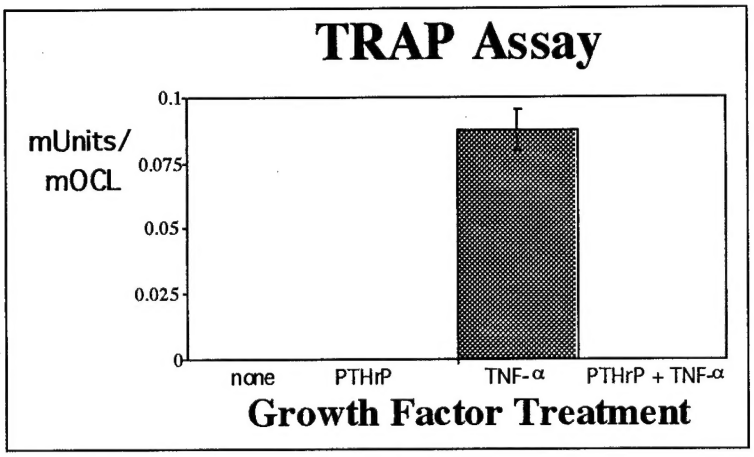
Isolated mouse osteoclast-like cells were generated in the presence of either vehicle or the indicated growth factor (PTHrP: 10 nM, TNF-α: 0.05ng/ml alone or combined), purified, and cultured for 24 hours on bone slices. The level of bone resorption was determined as we have previously described (1).



Conditioned media from the above cultures was analyzed for Cathepsin B enzyme activity levels as previously described (2). Activity was normalized to the number of TRAP positive multinucleated cells as determined above.



Conditioned media from the above cultures was analyzed for TRAP enzyme activity levels as previously described (2). Activity was normalized to the number of TRAP positive multinucleated cells as determined above.



KEY RESEARCH ACCOMPLISHMENTS:

- determined that breast cancer tumors which have metastasized to bone express GM-CSF, IGF II, TNF-α, and PTHrP mRNAs.
- determined that breast cancer tumors removed from bone secrete IGF-I, IGF II, GM-CSF, PTHrP, and TNF-α.
- discovered that osteoclasts that differentiate in the presence of TNF-α respond to continued TNF-α exposure with increased survival on the basis of viability and membrane phospholipid changes.
- GM-CSF has no effect on osteoclast lysosomal enzyme secretion whereas IGF II, TNF-α, and PTHrP stimulate osteoclast secretion of cathepsin B and tartrate resistant acid phosphatase.

REPORTABLE OUTCOMES

1 manuscript submitted to Cancer Research that has been provisionally accepted.

1 abstract to the 1997 American Society for Bone and Mineral Research annual meeting.

1 abstract to the 1998 American Society for Bone and Mineral Research annual meeting.

2 abstracts to the 1990 American Society for Bone and Mineral Research annual meeting.

funding applied for:

Grant Proposals in Revision for Resubmission:

National Institutes of Health: Prevention of Osteolysis. Planned resubmission date: 01/03/00 Dr. Merry Jo Oursler, P.I.

CONCLUSIONS:

We have discovered that breast cancer cells secrete a number of substances that alter osteoclast activity. Our final studies should discover if they work in concert to stimulate bone loss. Of importance is our discovery that tumor cells secrete TNF-a and that osteoclast differentiation in the presence of TNF-α utilize TNF-α as a survival factor. Our data have the potential to impact future therapies as this means that tumor cells stimulate osteoclast differentiation, increase the activity of each osteoclast, and further promote bone loss by extending the life span of each osteoclast. Thus, therapies could be designed to impact any one or more than one of these aspects of stimulation of osteolysis to decrease bone loss.

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Identification Of Breast Cancer Cell Line-Derived Paracrine Factors That Stimulate Osteoclast Activity.

Larry Pederson[1], Bent Winding[2], Niels T. Foged[2], Thomas C.Spelsberg[1], and Merry Jo Oursler[3]

[1]Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, 200 First St. SW Rochester, MN 55905. [2]Center for Clinical and Basic Research, Ballerup, Byvei 222 DK-2750, Ballerup, Denmark. [3]Departments of Biology, Medical Microbiology and Immunology, and Biochemistry and Molecular Biology, University of Minnesota, Duluth Minnesota, 55812

Running Title: Breast Cancer Cells Stimulate Osteoclast Activity

5 key words: osteoclast; osteolysis; metastasis; growth factors; bone resorption

Abstract

Metastatic breast cancer causes destruction of significant amounts of bone and, although bone is the most likely site of breast cancer metastasis, little is understood about interactions between tumor cells and bone resorbing osteoclasts. We have investigated the paracrine factors produced by breast cancer cells that are involved in increasing osteoclast activity. We have determined by immunoassay that the human breast cancer cell line MDA MB 231 (231) cultured in serum-free media secretes transforming growth factor type betas (TGF-ß1 and TGF-ß2), macrophage colony stimulating factor (M-CSF), granulocyte macrophage stimulating factor (GM-CSF), interleukins (IL-1 and IL-6), tumor necrosis factor alpha (TNF-α), insulin like growth factor II (IGF II), and parathyroid hormone related peptide (PTHrP). To determine which of these are involved in increased bone destruction, we have fractionated serum-free 231 conditioned media and measured these fractions for effects on osteoclast resorption activity using multiple activity assays. The pattern of responses was complex. Several fractions stimulated osteoclast resorption either by increasing the number of osteoclasts binding to the bone or by elevating the resorption activity of the individual osteoclasts. Other fractions inhibited osteoclast activity. Analysis of active fractions for the factors identified in the 231 conditioned media revealed that the presence of TNF-α and IGF-II was restricted to separate fractions that stimulated osteoclast resorption activity. The fractions that inhibited osteoclast resorption activity contained M-CSF, IL-6, TGF-ß2, and GM-CSF. No TGF-ß1 or IL-1 was detected in any of the active fractions. Our data supports the hypothesis that breast cancer cells modulate osteoclast activity using multiple regulatory factors that increased both the number of mature osteoclasts attached to the bone and the bone resorption activity of these individual osteoclasts. Once it is understood how metastatic breast cancer elevates osteoclast-mediated bone loss, effective therapies to slow the progression and/or prevent this bone loss will become possible.

Most women with breast cancer receive treatment that seeks to irradicate cancer cells from the breast. Twenty four percent of those patients with an apparent permanent elimination of cancer from the breast and a lack of evidence of skeletal metastases at the time of surgery will eventually develop signs or symptoms of breast cancer metastases involving the skeleton (1). This indicates that undetectable, microscopic bone metastases were present when the breast cancer was originally diagnosed and it underscores the importance of understanding how these microscopic breast cancer deposits in bone develop into clinically relevant tumors. Three events must occur before women with breast cancer develop the signs or symptoms of skeletal metastases. First, cancer cells must leave the breast, travel to bone, and occupy the osseous intramedullary compartment. Second, cancer cells within the osseous intramedullary compartment must induce bone destruction to provide space for tumor growth, and finally, clusters of cancer cells within the osseous intramedullary compartment must grow to form solid tumors. We are investigating the hypothesis that locally produced tumor-derived paracrine factors that are driving the debilitating bone loss associated with metastatic cancer.

The prevalence of bone metastasis in breast cancer patients is highlighted by the fact that, at the time of autopsy, 70% of the women who die from breast cancer show metastases to bone (reviewed in(2). Tumor cells travel to other parts of the body by altering their phenotype to exploit the blood vasculature and lymph system for transport and deposit in other tissues. Once the tumor cells arrive in bone, they can begin to grow and actively alter their environment to maximize growth. As this growth proceeds, the tumors stimulate the destruction of large amounts of bone at the site of the tumor. This focal loss of bone weakens the skeletal structure and usually results in considerable pain, decreased mobility, hypercalcemia, and significant levels of skeletal fracture. Once tumor cells are deposited and begin to grow in the bone, curative therapy is problematical. For most of these patients, the goals of treatment aim to alleviate discomfort and prevent pathological fractures. Current treatments enable control of tumors in the breast, and patient deaths are more likely due to metastatic cancer. Thus, therapies that limit tumor-driven bone destruction could greatly slow the progression of complications and suffering. Since a significant problem both in terms of patient suffering and in terms of promoting

tumor progression is the result of tumor-driven osteolysis, tumor stimulation of osteoclastic bone resorption is an important target in studies seeking for ways to slow tumor progression.

Multiple growth factors and cytokines have been reported to influence osteoclast differentiation (reviewed in (3, 4). These include macrophage colony stimulating factor 1 (MCSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukines 1 and 6 (IL-1 and IL-6), transforming growth factor betas (TGF-ß), Insulin like growth factors (IGFs), tumor necrosis factor alpha (TNF-α), and parathyroid hormone related peptide (PTHrP). Much less is known concerning the influences of these factors on the resorption activity of mature osteoclasts. Osteoclasts have been reported to express receptors for MCSF, TGF-ß, IGFs, IL-1, IL-6, and PTH/PTHrP (5-10). Thus, these factors could potentially impact the activity of the differentiated cells directly in addition to influencing differentiation. We have therefore sought to determine whether breast cancer cells stimulate osteoclast resorption directly and which growth factors secreted by these cells are candidate factors responsible for this stimulation.

METHODS

Collection of Breast Cancer Cell Line Conditioned Media: MDA MD 231 cells were obtained from ATCC (Rockland, MD) and subcultured in phenol red-free α Minimal Essential Media (αMEM) obtained from Gibco/BRL, Gaithersburg, MD, supplemented with 10 % fetal bovine serum at 37°C, 5% CO_2 until confluent. Cell monolayers were washed with sterile Phosphate Buffered Saline, pH 7.4 and placed in αMEM supplemented with 0.25% (wt/v) bovine serum albumin (BSA) obtained from Sigma Chemical Co., St. Louis, MO for 3 days. At the time of collection, cellular debris was removed by centrifugation, and aliquots were frozen at –70°C until analyzed.

Osteoclast Isolation and Culture: Osteoclasts were isolated from white leg horn hatchlings that are maintained on a low calcium diet for a period of 5 weeks (11). All animals were treated as humanely as possible and treatment followed the NIH and institutional guidelines for care and use of experimental animals. An osteoclast-directed monoclonal antibody, 121F (a gift from Dr. Philip Osdoby, Washington University, St. Louis, MO), coupled to immuno-magnetic beads obtained from

Dynal, Inc., was used to obtain cell populations that consists of at least 90% pure osteoclasts and 10% or less unidentified mononuclear cells (12). The purified osteoclasts exhibit all the phenotypic attributes of osteoclasts including multinucleation, attachment and ruffled border formation when cultured with bone particles, and the ability to attach and form resorption pits when cultured on slices of cortical bone. Osteoclasts were cultured in phenol red-free αMEM supplemented with 0.25% (wt/v) BSA as described for individual experiments (see figure legends and below).

Resorption Analyses:

Quantitative pit formation assay: Isolated osteoclasts were plated on 1mm^2 slices of bovine cortical bone. Bone slices were prepared as previously described (13). Samples were treated with vehicle or the indicated test substance as detailed in the figure legend. After 24 hours of culture, the slices were placed in 1% (v/v) paraformaldehyde in PBS. The number of osteoclasts per mm^2 slice was determined for each slice as follows: the fixed slices were rinsed with water and stained for tartrate resistant acid phosphatase (TRAP) activity using a Sigma histochemical kit. Osteoclasts were identified as stained multinucleated cells. The number of pits per osteoclast was determined following removal of the cells. The pits, resulting from osteoclast activity, were stained with toluidine blue, counted by reflected light microscopy, and expressed as the number of pits per osteoclast as previously described (13, 14).

Quantitative lysis of collagen by enzyme linked immunosorbent assay (ELISA): Osteoclasts were cultured on bone slices with either vehicle or the test substance as detailed in the figure legends. The conditioned media were harvested and the amount of antigenic collagen fragments released was determined as described (15).

Lysosomal enzyme assays: Cell pellet extracts and conditioned media were assayed. To standardize for relative cell number, the protein content of the solubilized cell pellet was determined using the BioRad protein detection system. TRAP activity was measured using an assay based on the work of Hofstee (16). The initial rate of hydorlysis of o-carboxy phenyl phosphate was determined by following the increase in absorbency at 300 nM resulting from the liberation of salicylic acid. One unit hydrolyses one micromole of o-carboxy phenyl phosphate per minute at 24° C, pH 5.0. The assay

was performed in the presence of 1 mM tartarate. Cathepsin B levels were measured by Na-CBZ-lysine p-nitrophenyl ester hydrolysis as measured by 520 nM absorbance as outlined by Barrett and Kirschke (17).

Preparation of Growth Factors: Recombinant human growth factors were purchased from R&D (Minneapolis, MN) and reconstituted in αMEM supplemented with 0.25% (wt/v) BSA at 1000-fold the concentration used in each experiment (see figure legends). Aliquots were stored at –70°C.

Conditioned media fractionation: Conditioned media was collected as outlined above and 1 ml loaded onto a Superdex 75 molecular sieve column (Pharmacia, Piscataway, N.J.) which has a functional separation range of 5-75 kD after pre-equilibration with α MEM using 3 bed volumes (150 ml) at 1 ml/min. Gel filtration separation of the sample is carried out with a flow rate of 0.5 ml/min. with a backpressure of 0.7 Mpa. Thirty 1 ml fractions were collected on ice and frozen immediately at -70°C until assayed for effects on osteoclast activity or growth factor quantitation.

Quantitation of growth factors and cytokines: Interleukin (IL) -1, IL-6, macrophage-colony stimulating factor (M-CSF). Granulocyte/ macrophage colony stimulating factor (GM-CSF), transforming growth factor beta(TGF-ß)1, TGF-ß2, and tumor necrosis factor-alpha (TNF-α) were quantitated using R&D Quantikine kits according to the instructions. Insulin-like growth factor –II (IGF-II) and parathyroid hormone related peptide (PTHrP) levels were analyzed by the method of de Leon et al. (18).

Statistical Analysis: Unless otherwise indicated in the figure legends, the results represent the mean +/- SEM of three separate experiments. The effect of treatment was compared with control values by one-way analysis of variance (ANOVA); significant treatment effects were further evaluated by the Fisher's least significant difference method of multiple comparisons in a one-way analysis of variance. Tests were carried out using Apple software, obtained from Statview II, Abacus Concepts, Inc., Cupertino, CA.

RESULTS

Breast Cancer Cell Line Conditioned Media Studies: Initially, we surveyed conditioned media from several well-characterized breast cancer cell lines for their effects on osteoclast resorption activity. As

demonstrated in Figure 1, conditioned media from each of these cell lines stimulated bone resorption, although the stimulatory level varied with the cell line. For subsequent studies, we have focused our studies on the cell line MDA MB 231 as this cell line has proven to cause osteolytic lesion in an *in vivo* animal model (19). In order to estimate the total volume of bone resorbed when osteoclasts are cultured in the presence of MDA MB 231 cell conditioned media, we have utilized a newly-developed assay that quantitates the amount of collagen peptide released. Osteoclasts were cultured on bone slices and treated with a series of dilutions of MDA MB 231 cell conditioned media (Figure 2A). This analysis revealed that there was a dose-dependent effect of the conditioned media on osteoclast activity. Interestingly, the response was biphasic, with a maximal effect at a 0.1% dilution of the conditioned media and an inhibitor effect at the highest dose. Using the pit formation assay, a biphasic effect of the conditioned media was observed, but the peak stimulation in the number of pits per osteoclast was at a dilution of 1% conditioned media (Figure 2B). Again, the highest dilution was inhibitory. A similar pattern emerged when the number of osteoclasts per bone slice was assessed with the peak concentration at 0.1% conditioned media (Figure 2C). Having ascertained that MDA MB 231 cells produced a substance or substances that stimulated osteoclast activity, the conditioned media was assayed for the presence of a number of cytokines and growth factors (Table I). Significant levels of IL-1, IL-6, M-CSF, GM-CSF, TGF-ß1, TGF-ß2, TNF-α, IGF-II, and PTHrP were measured in the conditioned media.

Identification Of Candidate Stimulatory Factors: The above list contained many factors that could be working either alone or in combinations to stimulate osteoclast activity. To further define the list of candidate osteoclast stimulatory factors, MDA MB 231 conditioned media was passed over a molecular sieve column under non-denaturing conditions. The resultant fractions were sterile filtered and assessed for effects on osteoclast activity. The pattern of effects on the total amount of collagen peptide released into the media suggested that there were regions that stimulated resorption and, interestingly, regions that repressed osteoclastic activity (Figure 3A). When the number of pits per osteoclast was examined, there were several fractions which stimulated bone resorption, but no regions which appeared to inhibit the number of pits formed per cell (Figure 3B). When the number of osteoclasts per slice was examined, fractions which had elevated collagen releasing effects but no

effect on the number of pits per slice contained more cells per slice (Figure 3C). Similar to the pattern observed when the amount of collagen peptide released was determined, there were fractions that had fewer osteoclasts per slide than control cultures. These assays have indicated that there were stimulatory and inhibitory conditioned media fractions. Both stimulatory and inhibitory fractions were assayed for the presence of the same growth factors that were identified in Table I. As detailed in Table II, TNF-α and IGF-II were present in fractions that stimulated resorption. GM-CSF, M-CSF, IL-6, TGF-ß2, and PTHrP were present in the fractions inhibited osteoclast activity. No IL-1 or TGF-ß1 was measured in any of the active fractions. All of the active fractions were examined for cytokine and growth factor levels, and several of the active fractions contained no detectable levels of any of the factors examined.

Effects of Growth Factors on Osteoclast Activity: We examined the effects of the above identified factors on osteoclast resorption activity (Figure 4). IGF-II, PTHrP, and TNF-α each stimulated resorption activity by all of the parameters measured here. TGF-ß2 stimulated the number of pits per osteoclast and the number of osteoclasts per slice, but the total volume of collagen peptide released was not significantly altered with treatment. In contrast, treatment with GM-CSF significantly inhibited resorption activity as measured by determining the number of pits per osteoclast while there appeared to be no significant effects on the number of osteoclasts per slice or on the total amount of collagen released. IL-6 treatment increased the number of pits per slice yet there was no significant effect on either the amount of collagen peptide released or on the activity per osteoclast. We were unable to detect any significant effect of M-CSF on resorption activity during the 24-hour treatment period by any of the criteria measured here. Having measured effects of these growth factors on resorption activity, we next determined whether the factors likewise influenced lysosomal enzyme secretion. As demonstrated in Figure 5, IGF-II, PTHrP, TNF-α, TGF-ß2, and IL-6 stimulated cathepsin B and TRAP secretion when results were normalized for respective sample cell numbers. Treatment with GM-CSF, M-CSF had no effect on the secretion per osteoclast of cathepsin B but inhibited TRAP secretion.

Effects of growth factor combinations on osteoclast activity: Having assessed the effects of treatment with individual growth factors on osteoclast resorption and lysosomal enzyme activity, we explored

the influences of the combinations of growth factors present in the conditioned media fractions that had either stimulatory or inhibitory effects (Table III). The combination of IGF-II, TGF-ß2, PTHrP, and TNF-α approached the stimulatory level of 0.1% conditioned media with respect to effects on bone resorption and lysosomal enzyme secretion. Interestingly, the combination of GM-CSF, M-CSF, IL-6, PTHrP, and TGF-ß2 inhibited these same parameters. Combining stimulatory IGF-II and TNF-α with these inhibitory factors resulted in stimulatory activity, but the level of stimulation did not approach that of the diluted conditioned media.

DISCUSSION

The data presented here demonstrate that breast cancer cells secrete multiple growth factors that have the ability to stimulate osteoclast-mediated bone loss. We have shown that all of the cell lines examined secrete factors that stimulate osteoclast resorption activity. For the remaining studies, we elected to examine the MDA MB 231 cell conditioned media as these have proven to be highly metastatic to bone using an animal model system pioneered by Nakai et al. (19). We have examined three different resorption parameters for these studies. Quantitation of the total amount of bone removed was achieved by determining the amount of collagen peptide released into the media during the resorption process. This assay detects the total amount released whether it is due to increased numbers of osteoclasts, increased number of pits generated by each osteoclast, or increased pit size. Analysis of the number of pits per osteoclast indicated the activity per cell and the calculation of the number of osteoclasts per bone slice indicated the number of cells that were present. Changes in this last parameter could be due to a number of different effects including decreased apoptosis or increased binding to bone. Resolution of the mechanisms by which the number of osteoclasts present were altered is not revealed by these studies and remains to be resolved with further experimentation. The conditioned media effects were seen at surprisingly low concentrations and were biphasic. Our studies revealed that all of the measures of osteoclast activity including the total amount of bone removed, the activity of each osteoclast, and the number of osteoclasts bound to the bone exhibited this biphasic response. There are several possible reasons for this: depletion of important factors by using spent media or a toxic metabolic waste build up. Depletion of important factors is unlikely given the identification of inhibitory fractions after chromatography in fresh media. Any accumulated toxic

metabolic products would elute as small molecules in late fractions. The inhibitory fractions were in mid range (estimated sizing between 40 to 100 kDa), indicating that this is not a likely explanation either.

Not surprisingly, there were many growth factors and cytokines present in the conditioned media. To better define the growth factors in the conditioned media that were active in stimulating osteoclast activity, the media was fractionated using an approach that was not disruptive to native protein conformations and interactions. Analysis of these fractions with the three different resorption activity measurements has revealed an interesting pattern of responses which varies according to which resorption criteria was examined. As outlined in Table IV, fractions 3, 4, and 5 increased the total amount of bone removed and the activity of each osteoclast. In these fractions, the number of osteoclasts per slice was decreased; thus the stimulation in resorption activity is likely to be due to the elevation in activity of the individual osteoclast. In contrast, fractions 6 and 7 significantly stimulated the number of cells per slice whereas there was no significant effect on the activity per osteoclast. Thus, the elevation in the amount of total bone excavated is likely to be, at least in part, the result of an increase in the number of cells on the bone slices, counterbalancing the lack of any effect on the activity per osteoclast. As fraction 6 contained TNF-α, we examined the effects of TNF-α on osteoclast numbers. The data presented here supports that TNF-α elevates the number of osteoclasts found on bone after short-term treatment and we are currently pursuing the mechanisms of this effect. Fractions 9 and 10 caused an increase in the activity of each osteoclast while the number of osteoclasts per slice was decreased. These combined to slightly stimulate bone resorption. Fraction 10 contains IGF-II and our data demonstrate that the major influence of IGF-II is on the activity of individual osteoclasts, supporting that IGF-II may be important in tumor-driven stimulation of osteoclast activity. When the amount of collagen peptide released was examined, fractions 23 and 24 stimulated bone resorption. In these fractions, there was no effect observed on the activity level of the osteoclasts and very small stimulation in the number of osteoclasts present. It may be that the elevation in collagen peptide released in these samples was due to each osteoclast generating a larger resorption pit. Interestingly, fractions 11 through 20 inhibited the amount of bone removed. In these fractions, the activity of each osteoclast was stimulated while the number of osteoclasts was depressed. There are

many growth factors present in these fractions, and our data demonstrate that the interactions of these growth factors results in repressed bone resorption and lysosomal enzyme secretion. The observed decrease in total bone loss may be the result of a decrease in osteoclast binding, an elevation in apoptosis, or might also be due to shallower pits being generated. The precise nature of this observation and the interactions of these growth factors will also require further study. This effect of repressing resorption by these fractions may at least in part explain the bi-phasic nature of the dilution curve observed above. If substances are present in the media that repress resorption, the higher concentrations of them could repress activity in spite of the presence of the stimulatory agents.

We examined all of these fractions to determine the presence of IL-1, IL-6, M-CSF, GM-CSF, TGF-ß1, TGF-ß2, TNF-α, IGF-II and PTHrP. In the stimulatory fractions, we detected TNF-α in fraction 6 and IGF-II in fraction 10. None of the other fractions, whether stimulatory, inhibitory contained these factors. As indicated in Table II, some of the inhibitory fractions contained GM-CSF, M-CSF, IL-6, TGF-ß2, and PTHrP. Other inhibitory fractions contained no detectable levels of the factors examined. This is intriguing and we are currently pursuing the content of these fractions with more extensive studies. None of the fractions contained detectable levels of TGF-ß1 or IL-1, suggesting the possibility that these factors were diluted by the fractionation to sufficiently to be below the detection limits of the assays (TGF-ß1: < 7pg/ml; IL-1: 0.5 pg/ml) or they were present in untested, therefore inactive, fractions.

Since several of the factors present in the inhibitory fractions have been shown to stimulate osteoclastic resorption we examined whether the factors identified in the stimulatory fractions were capable of stimulating osteoclasts in our system. Our studies revealed that human IGF-II and TNF-α both stimulated the activity of the avian osteoclasts. Since these were identified in two of the stimulatory fractions, it seems likely that these factors are breast cancer-derived factors that are involved in stimulating osteoclast-mediated bone resorption. Hou et al. have demonstrated that purified rabbit osteoclasts have IGF-I receptors, bind IGF-I with high affinity, and respond to IGF-I treatment with decreased apoptosis (7). In contrast with these finding, others have shown that IGFs have either no effect or stimulate only if osteoblasts are present (20, 21). TNF-α stimulates osteoclast differentiation, but the effects on mature cells have not been extensively studied (3, 4). TNF-α receptor 1 knockouts

appear to have normal bone, suggesting that TNF-α has little role in normal bone development, which does not preclude a role in pathological bone loss (22). IGF-II, TGF-ß, PTHrP, and TNF-α each individually stimulated osteoclast activity. Addition of these factors together (in concentrations similar to that found in diluted 231 conditioned media) stimulated osteoclast activity to a level approaching that of the diluted conditioned media.

Interestingly, the effects of the factors identified in the inhibitory fractions were more complex. Individually, several of the factors stimulated the activity of the isolated osteoclasts while neither GM-CSF nor M-CSF stimulated activity. Indeed, GM-CSF decreased the activity of individual osteoclasts. There is little data available on GM-CSF effects on mature osteoclast activity, but M-CSF has been implicated in suppressing apoptosis (23). IL-6 significantly increased the number of osteoclasts per slice while having no effect on the activity per cell or the amount of collagen peptide released. Since the major effects of IL-6 appear to on osteoclast differentiation and we are examining highly purified mature osteoclasts, a lack of effect of IL-6 on resorption activity is not surprising (24). In seeming contradiction to these results, it has been shown that mature osteoclasts have IL-6 receptors and that IL-6 reverses calcium-induced decreases in bone resorption (25). Direct effects of PTHrP on osteoclast activity have not been reported, but PTH, which utilizes the same receptor, appears to directly alter F actin distribution and cytosolic pH (8, 26). TGF-ß influences on osteoclast activity are somewhat mixed, with demonstrations of stimulation and inhibition of resorption and also a stimulation of apoptosis (27, 28). Thus, studies of direct growth factor effects on resorption activity are in their infancy as highly purified authentic cells are not routinely used for these studies and receptor identification studies are just now being undertaken. In our studies, the combination of the factors found in the fractions that decreased bone resorption (GM-CSF, M-CSF, IL-6, PTHrP, and TGF-ß) were inhibitory, supporting that the inhibitory factors present were able to overcome the stimulatory effects of some of the components of the mixture. When IGF-II and TNF-α were added to this inhibitory mixture, there was stimulation of resorption activity, but the level did not approached the stimulatory effects of the conditioned media. This leads us to conjecture that there are other stimulatory factors being secreted by the tumor cells that we have not identified. This possibility is

strengthened by the presence of stimulatory fractions in which we were unable to detect the factors we are studying.

Taken together, the data presented here demonstrate that metastatic breast cancer tumors are likely to produce multiple factors that have diverse effects on osteoclast bone resorption activity. The effect of some of the secreted factors suppressed bone resorption was unexpected, but our data clearly show that the overall effect of the combination of inhibitory and stimulatory factors was stimulatory. This is based on both the conditioned media studies and the effects of combined stimulatory and inhibitory purified growth factor studies. These data support that IGF-II and TNF-α are likely to be key factors secreted by metastatic breast cancer tumors responsible for stimulating bone resorption activity.

ACKNOWLEDGEMENTS

The tireless work of Ryan Sportel in the resorption and lysosomal enzyme assays is gratefully acknowledged. This research was supported by Department of the Army grant #DAMD17--1-7157, National Institutes of Health grant #R21 CA 65800 to the Mayo Clinic Cancer Center, and The Whiteside Institute for Clinical Research.

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TABLE I

GROWTH FACTOR/CYTOKINE ANALYSIS OF CONDITIONED MEDIA

FACTOR	CONCENTRATION (pg/ml)
IL-1	31.7 ± 3.6
IL-6	45.2 ± 7.5
M-CSF	14,900 ± 1,245
GM-CSF	152.2 ± 54
TGF-β1	1,237.8 ± 97.1
TGF-β2	6,895.4 ± 2437
TNF-α	31.1 ± 5.3
IGF-II	1,000 ± 50
PTHrP	35,000 ± 7,000

Conditioned media from MDA MB 231 cells were analyzed intriplicate for the indicated growth factors. Analysis was performed from 3 separate batches of conditioned media and these results are representative of these analyses. The results are the mean +/- SEM of the replicates.

TABLE II

GROWTH FACTOR ANDCYTOKINE ANALYSIS OF FRACTIONS

FRACTION#	FACTOR(S) (pg/ml)
6	TNF-α (14+/-1.1)
10	IGF II (12 +/-2.3)
13	M-CSF (362+/-68), IL-6 (34+/-7), PTHrP (7,000), TGF-ß2(18,260+/-75)
14	GM-CSF (128+/-18), M-CSF (792+/-530) IL-6 (112+/-14, TGF-ß2 (41,590+/-1276)
15	GM-CSF (297+/-13), M-CSF (3,168+/-184), IL-6 (924+/-17), TGF-ß2 (6,400+/-527), PTHrP (5,000)
16	GM-CSF (86+/-17), IL-6 (496+/-21)

All active fractions of the MDA MB 231 conditioned media were analyzed in triplicate for all of the growth factors listed in Table I. The fractions listed above were positive for the indicated growth factors and the concentrations (mean +/- SEM of three replicates) are given in parentheses. This experiment was repeated a total of three times and these results are representative of the levels detected.

TABLE III

EFFECTS OF GROWTH FACTOR COMBINATIONS ON OSTEOCLAST ACTIVITY

TREATMENT	COLLAGEN nM	PITS/OC	# OC	CATH B	TRAP
VEHICLE	1.7 ± .2	0.46 ±09	43 ± 7	34 ± 9	1.5 ±.7
0.1% CONDITIONED MEDIA	9.2 ± .4**	0.87 ±.10*	88 ±12*	299 ±26**	8.2 ±.4**
IGF-II+TGF-ß+PTHrP +TNF-α	9.7 ± .5**	0.91 ±.14*	103 ± 4**	258 ±27**	8.6 ±.4**
GM-CSF+M-CSF+IL-6+PTHrP+TGF-ß	0.4 ± .4 *	0.27 ±.07*	39 ± 9	12 ± 8 *	0.8 ±.2
TNF-α + IGF-II GM-CSF+M-CSF+IL-6+PTHrP+TGF-ß	5.1 ± .3**	0.36 ±.09*	55 ± 7**	87 ±16 **	2.3 ±.3**

Isolated osteoclasts weretreated with vehicle, conditioned media, the growth factors which individually stimulated osteoclast activity in the concentrations as indicated in Figure 4, and/or the growth factors which are present in the inhibitory fractions of conditioned media as detailed in Table II (GM-CSF+M-CSF+IL-6+PTHrP+TGF-ß). The cultures were analyzed as detailed in the Methods section. The experiment was repeated 3 times and the data are from one of these experiments and represent typical results. Data are the Mean +/- the SEM of the replicates from one experiment. *P<0.01; **P<0.001 comparing vehicle to treatment.

TABLE IV

COMPARATIVE ANALYSIS OF CONDITONED MEDIA FRACTIONS

FRACTION #	TOTAL VOLUME OF BONE REMOVED	ACTIVITY/CELL	OSTEOCLAST #	REGULATORY FACTOR CAUSING RESPONSE
3-5	↑	↑	↓	↑ OC ACT/CELL
6,7	↑	↔	↑	↑ # OCs BOUND
9,10	slight ↑	↑	↓	↑ COUNTER BALBNCE OC ACT/CELL & # OCs BOUND
11-20	↓	variable ↑	variable ↓	↓ # OC BOUND ? PIT VOLUME
23,24	↑	↔	slight ↑	↑ ? PIT VOLUME

With reference to the fractions numbers indicated on the left, the effects of treatment with the conditioned media in the indicated fraction on the total volume of bone removed (obtained by collagen peptide release measurement), the acitivty per cell (obtained from the pits/osteoclast measurement), and the osteoclast number (obtained from the osteoclast per slice measurement) provide some indication of the predominant effect of the conditioned media fraction on bone resorption. Assuming that the amount of bone removed indicates the overall effect on bone resorption activity, it is possible to project the likely regulatory effects as indicated in the last column on the right.

FIGURE LEGEND

Figure 1: Osteoclast Responses To Selected Breast Cancer Cell Line Conditioned Media: Isolated osteoclasts were cultured with the indicated concentration of the following tumor cell line conditioned media: MDA MB 231 (231), MDA MB 435 (435), MCF-7, and T47D or control (CONT). Analysis was done following 24 hours of culture. The number of pits formed per osteoclast per 1 mm^2 bone slice was determined as described in the Methods section. The experiment was done in triplicate and the results are presented as the mean +/-S.E.M. *$p<0.05$.

Figure 2: Effects of Human Breast Cancer Cell line Conditioned Media on Osteoclast Resorption Activity. Isolated osteoclasts were cultured with the indicated concentration of MDA MB 231 tumor cell line conditioned media. Analysis was done following 24 hours of culture. (A): The amount of collagen peptide released into the media. (B): The number of pits formed per osteoclast per 1 mm^2 bone slice. (C): The number of osteoclast per 1 mm^2 bone slice. Each experiment was done in triplicate and the results are presented as the mean +/-S.E.M. *$p<0.05$.

Figure 3: Size Fractionation of MDA MB 231 Conditioned Media. Conditioned media (0.5 ml) was fractionated and added to aliquots of freshly isolated osteoclasts on slices of bone following filter sterilization. Analysis was done following 24 hours of culture. Results are presented for (A): The amount of collagen peptide released into the media; (B): the number of pits formed per osteoclast per 1 mm^2 bone slice; (C): the number of osteoclasts per 1 mm^2 bone slice. Each experiment was done in triplicate and the results are presented as the mean +/- S.E.M. *$p<0.001$.

Figure 4: Analysis of Selected Factor Effects on Bone Resorption Activity. Isolated osteoclasts were cultured with either vehicle (media with 0.25% BSA) or the indicated factors at the following concentrations: GM-CSF: 0.15ng/ml; IGF-II: 10 pg/ml; PTHrP: 5 ng/ml; TNF-α: 15 pg/ml; TGF-$ß_2$: 50 ng/ml; M-CSF: 3 ng/ml; IL 6: 500 ng/ml for 24 hours; The concentration was selected with reference to Table II. Results are presented for (A): The amount of collagen peptide released into the media; (B): the number of pits formed per osteoclast; and (C): the number of osteoclasts per 1 mm^2 bone slice. The experiment was done a total of three times and these are representative results. *P<0.05; **P<0.01 relative to control.

Figure 5: Analysis of Selected Factor Effects on Lysosomal Enzyme Secretion. Isolated osteoclasts were cultured with either vehicle (media with 0.25% BSA) or the indicated factors at the following concentrations: GM-CSF: 0.15ng/ml; IGF-II: 10 pg/ml; PTHrP: 5 ng/ml; TNF-α: 15 pg/ml; TGF-$ß_2$: 50 ng/ml; M-CSF: 3 ng/ml; IL 6: 500 ng/ml for 24 hours; The concentration was selected with reference to Table II. Results are presented for (A): Cathepsin B activity in the conditioned media and (B): TRAP activity in the conditioned media. Assays were performed on 7 slices/treatment. The experiment was done a total of three times and these are representative results. Results are mean +/- S.E.M. *p<0.01.

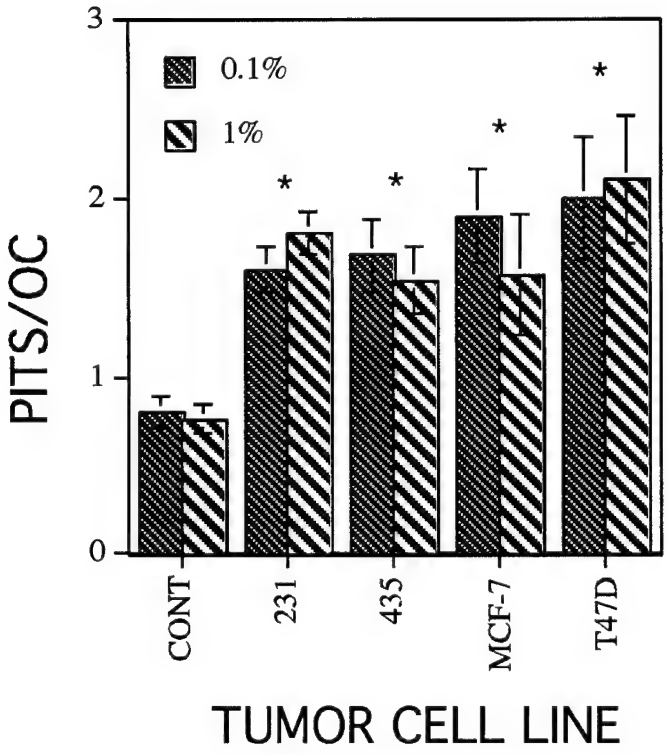
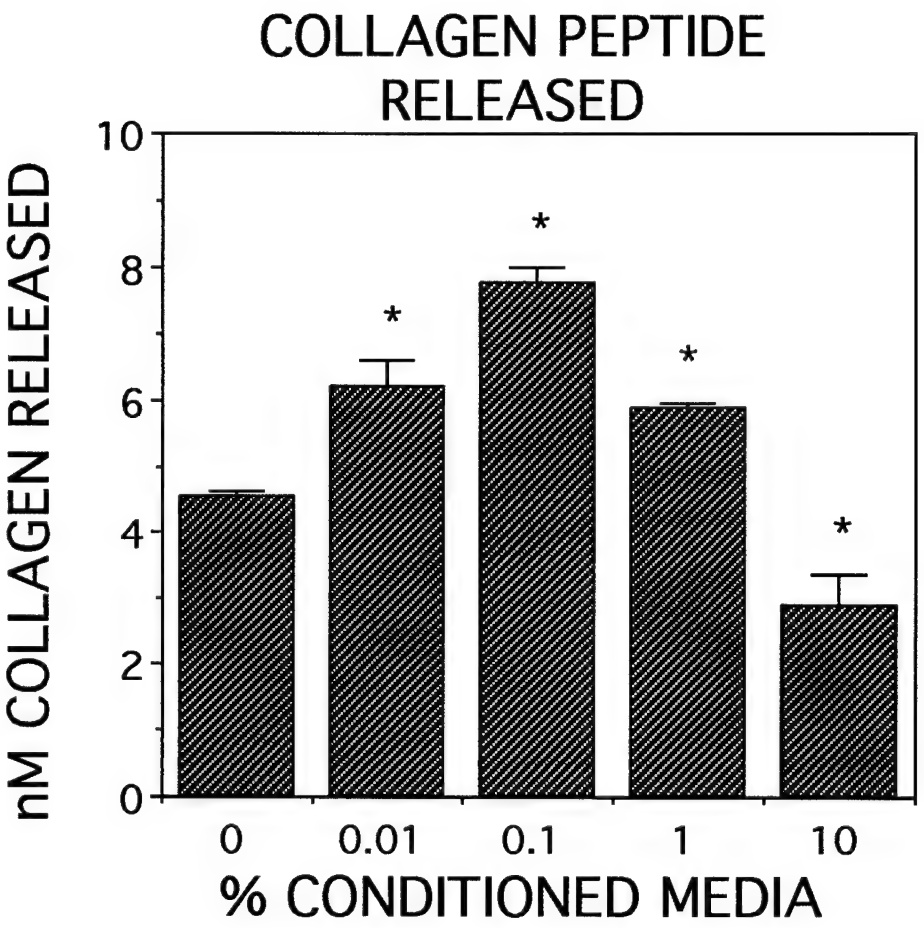


Figure 1

A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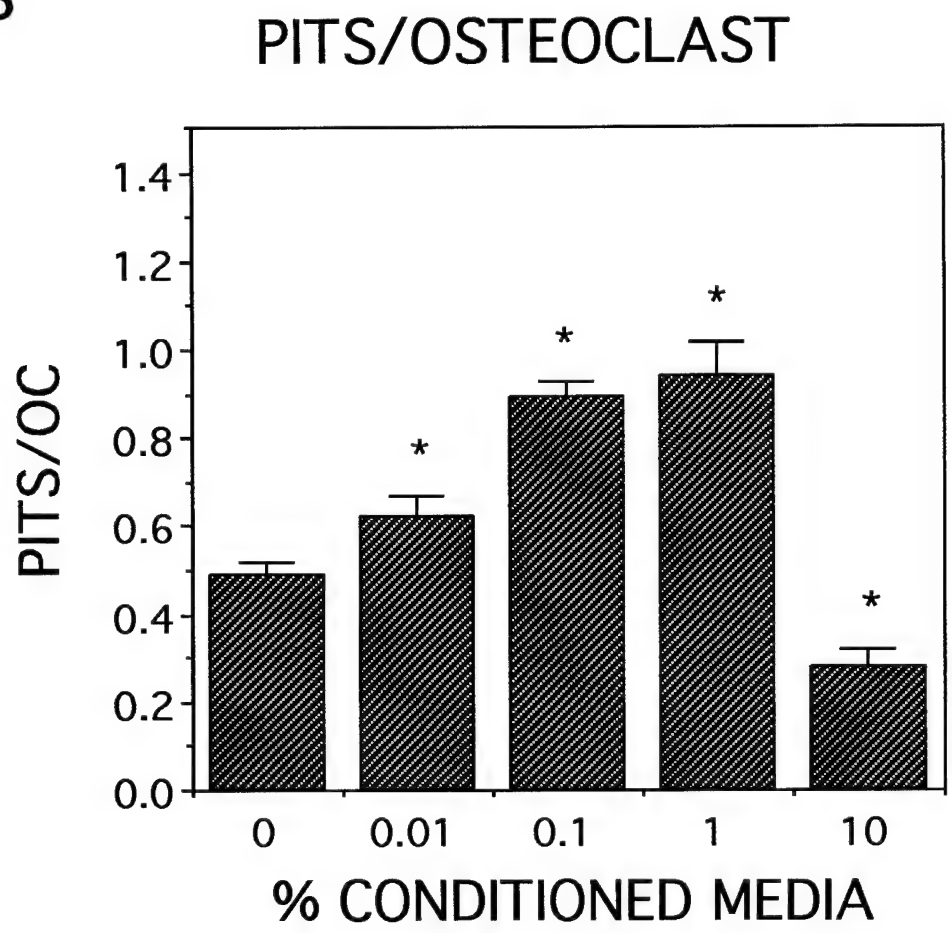
% CONDITIONED MEDIA

Figure 2A

B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% CONDITIONED MEDIA

Figure 2B

C

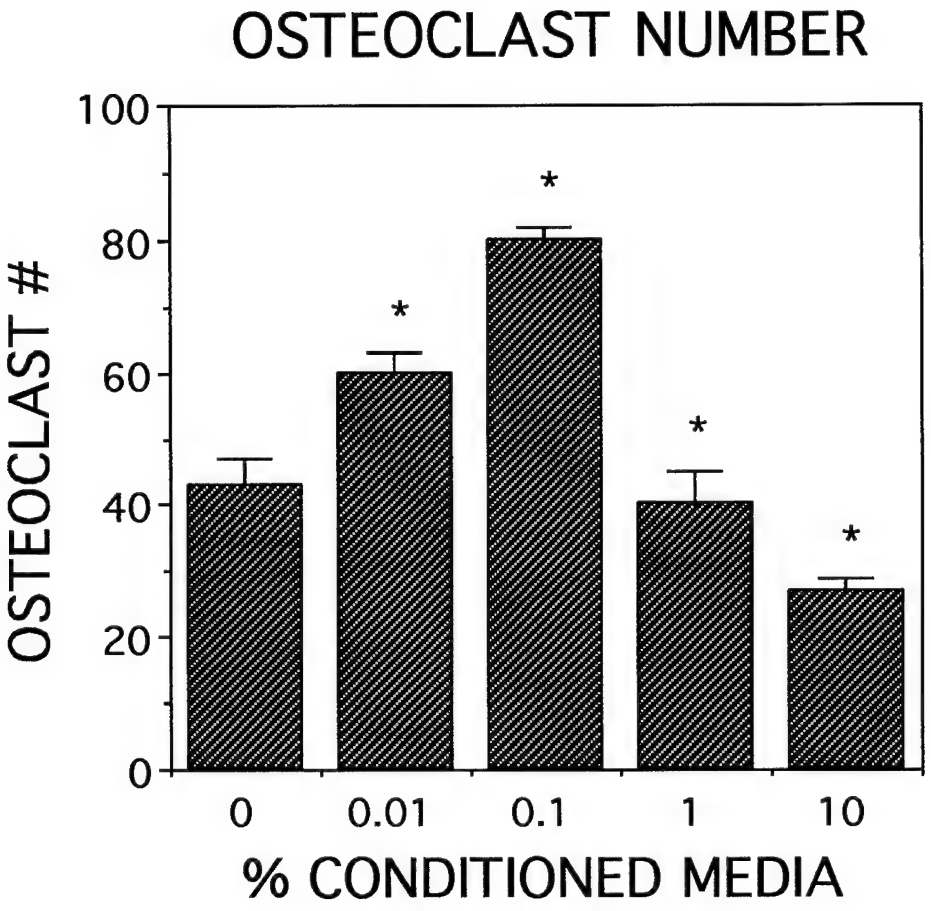
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Figure 2C

A

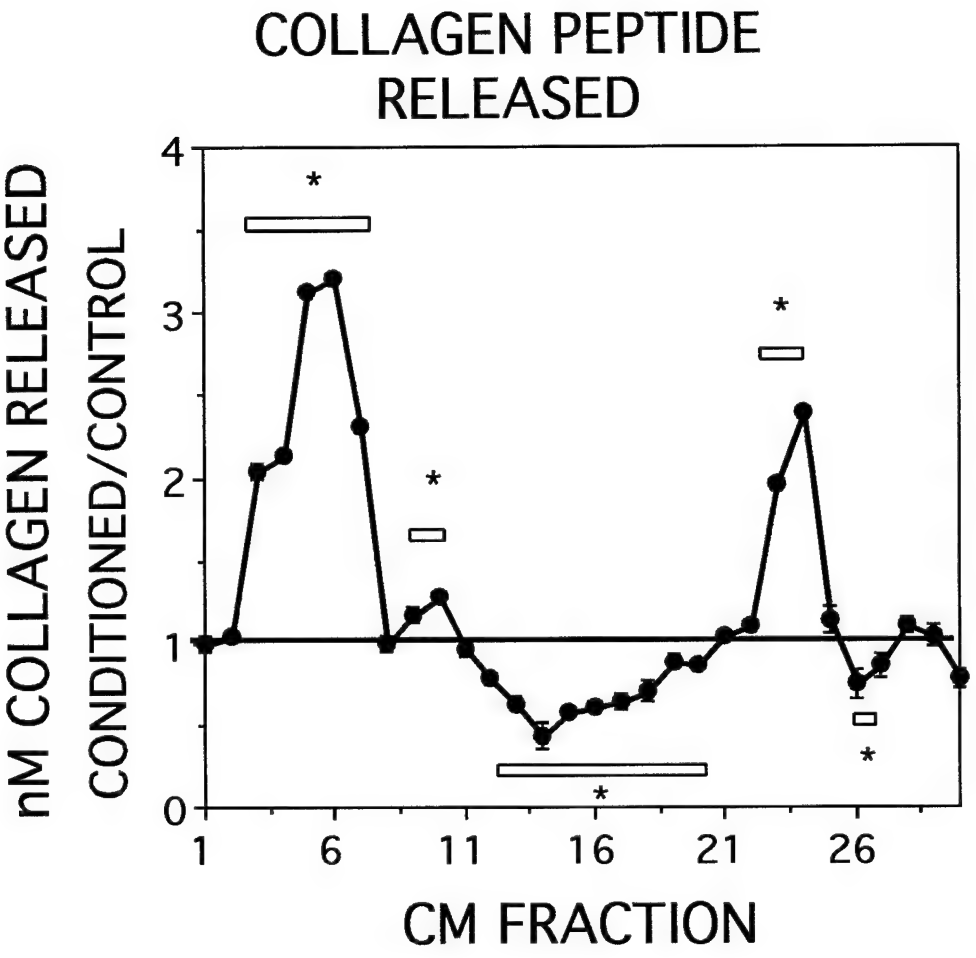
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Figure 3A

B

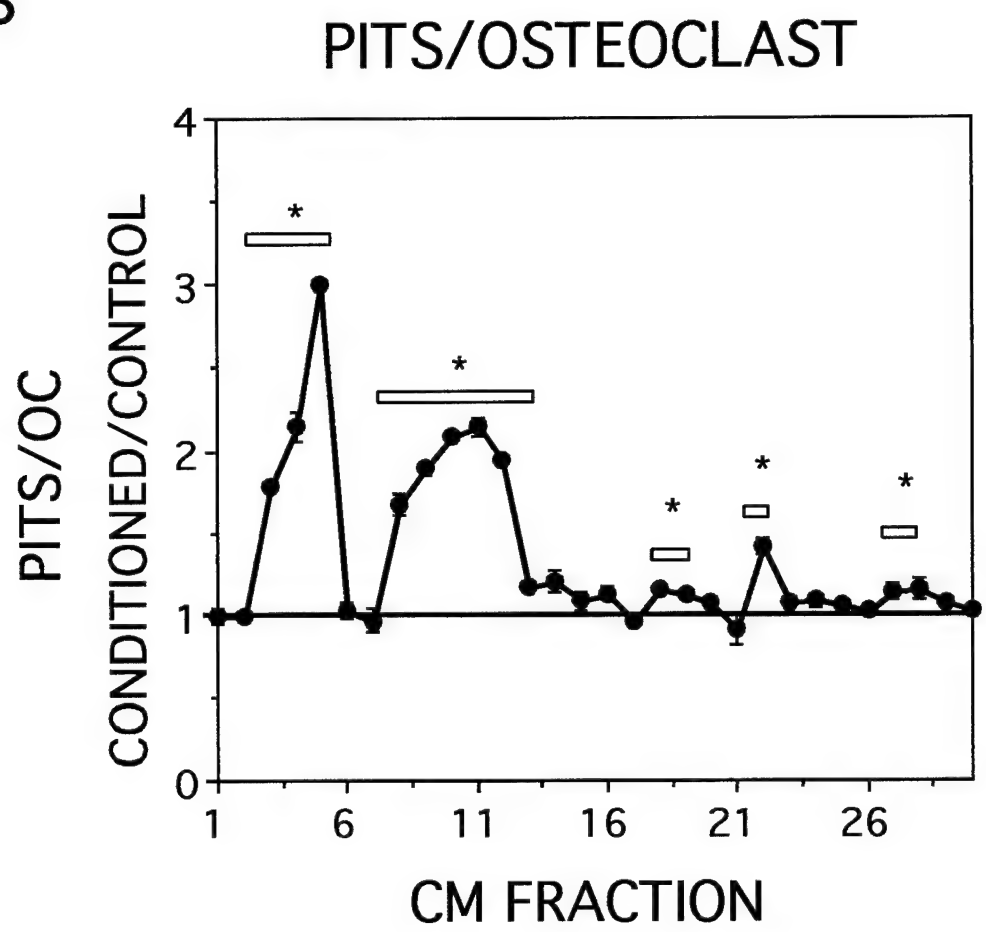
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Figure 3B

C

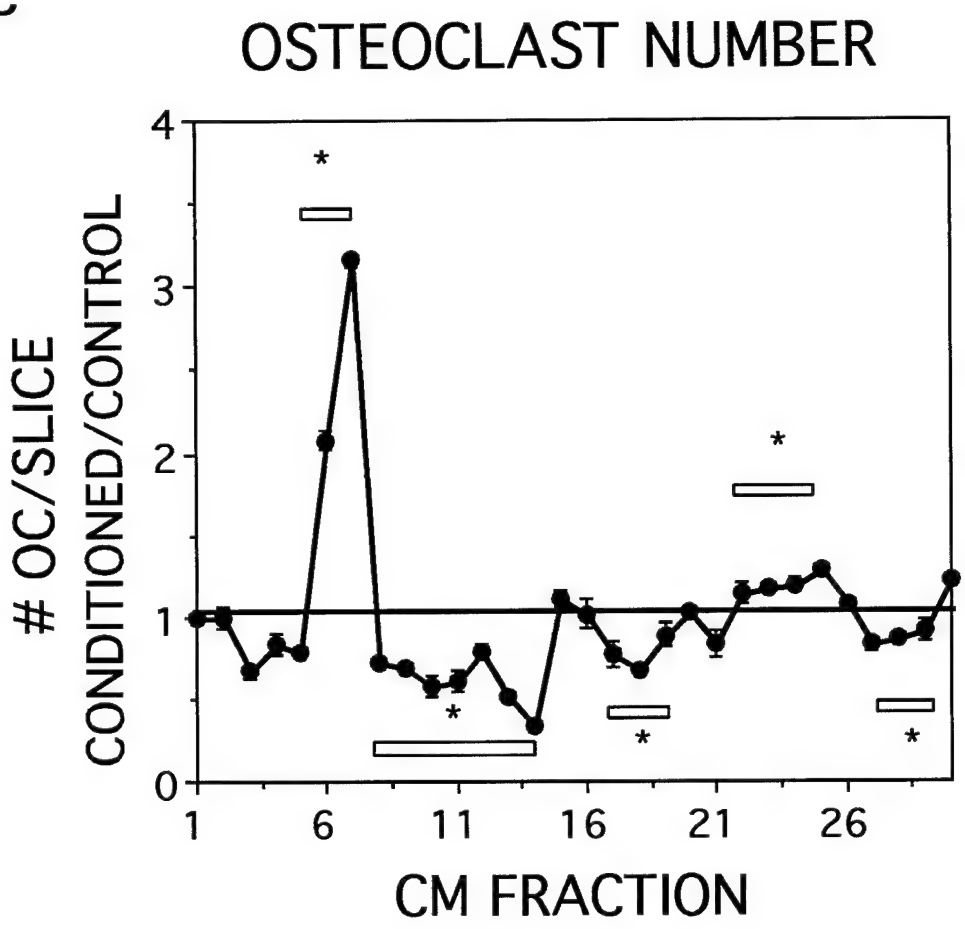
OSTEOCLAST NUMBER
OC/SLICE
CONDITIONED/CONTROL
0
1
2
3
4
1
6
11
16
21
26
CM FRACTION
*
*
*
*
*

Figure 3C

A

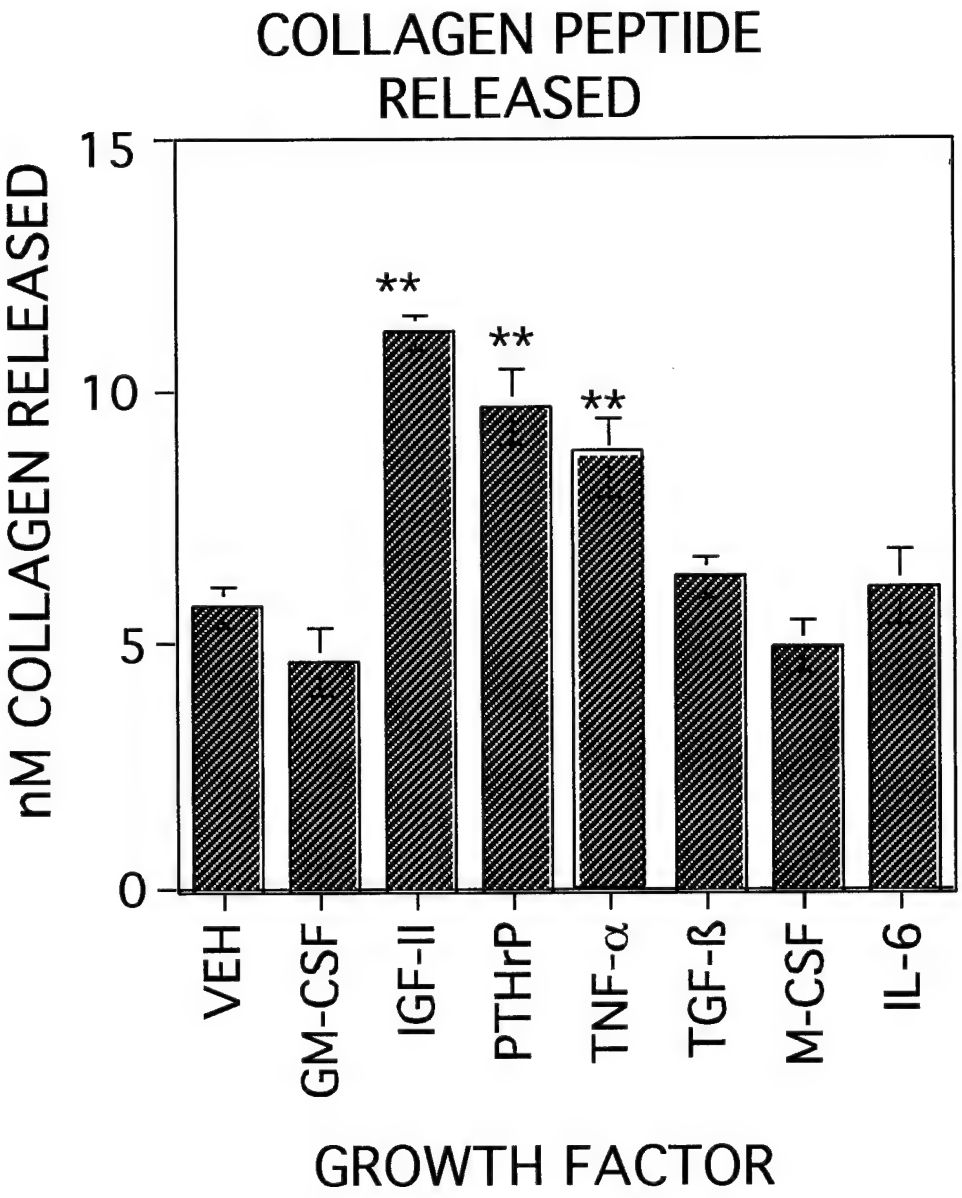
COLLAGEN PEPTIDE RELEASED
nM COLLAGEN RELEASED
15
10
5
0
**
**
**
VEH
GM-CSF
IGF-II
PTHrP
TNF-α
TGF-ß
M-CSF
IL-6
GROWTH FACTOR

Figure 4A

B

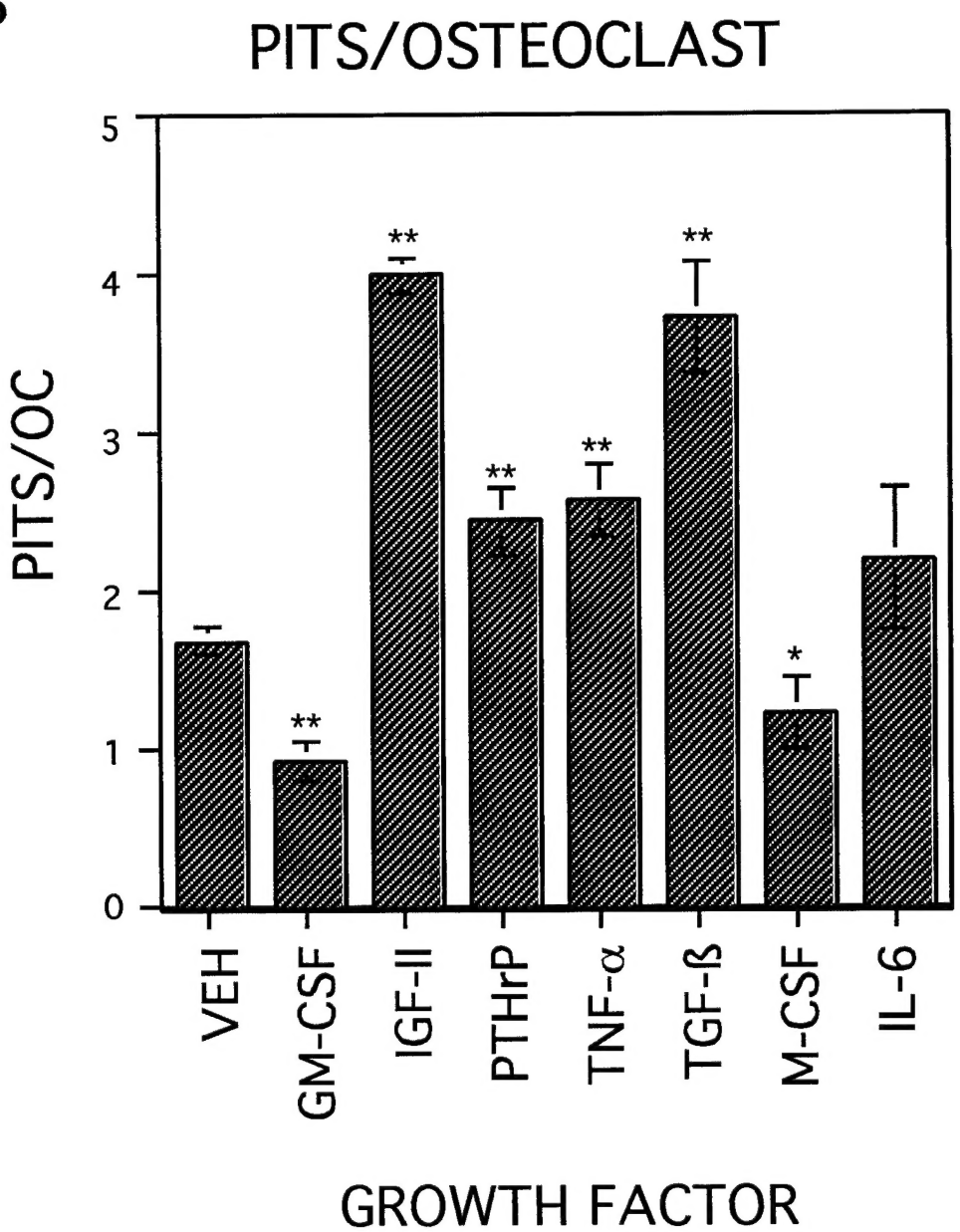
PITS/OSTEOCLAST
PITS/OC
5
4
3
2
1
0
**
**
**
**
*
VEH
GM-CSF
IGF-II
PTHrP
TNF-α
TGF-ß
M-CSF
IL-6
GROWTH FACTOR

Figure 4B

C

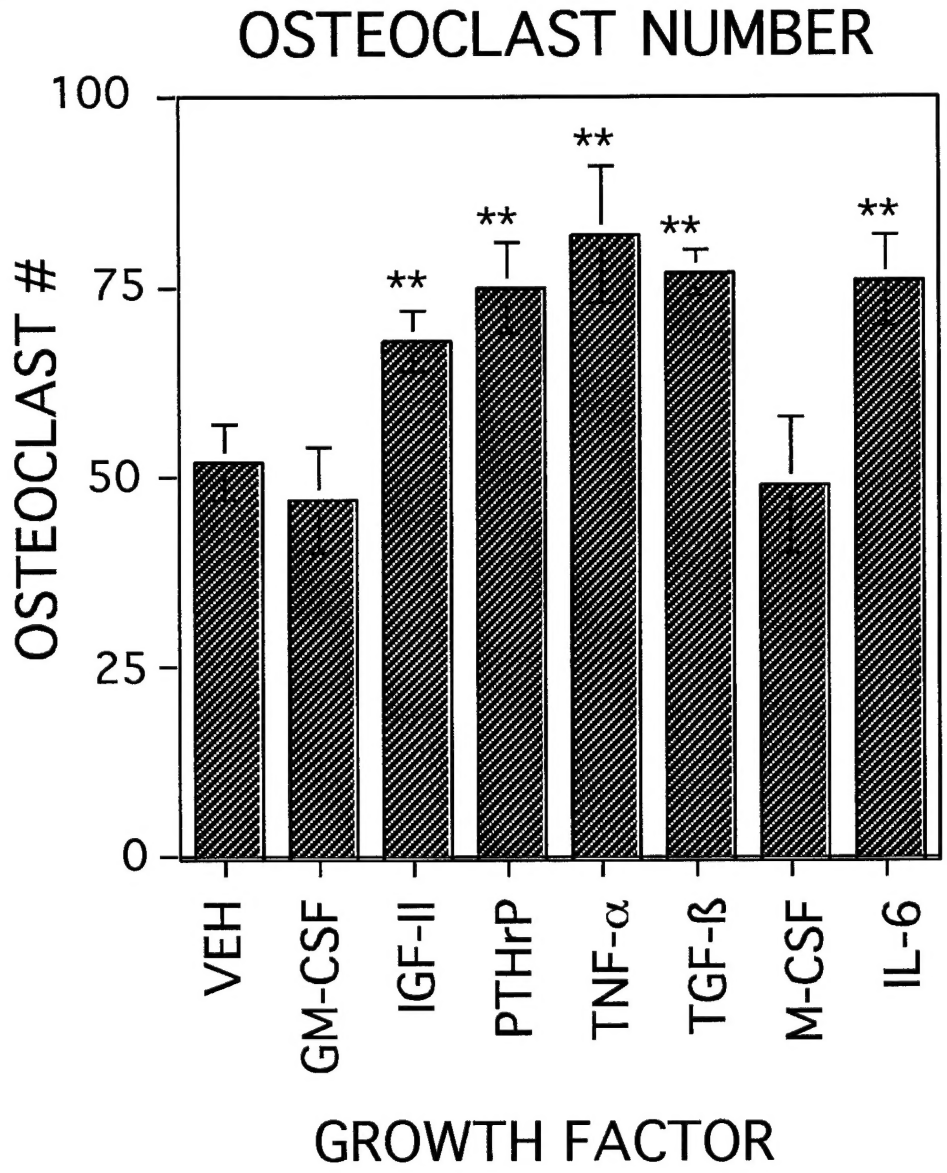
OSTEOCLAST NUMBER
OSTEOCLAST #
100
75
50
25
0
**
VEH
GM-CSF
IGF-II
PTHrP
TNF-α
TGF-ß
M-CSF
IL-6
GROWTH FACTOR

Figure 4C

A

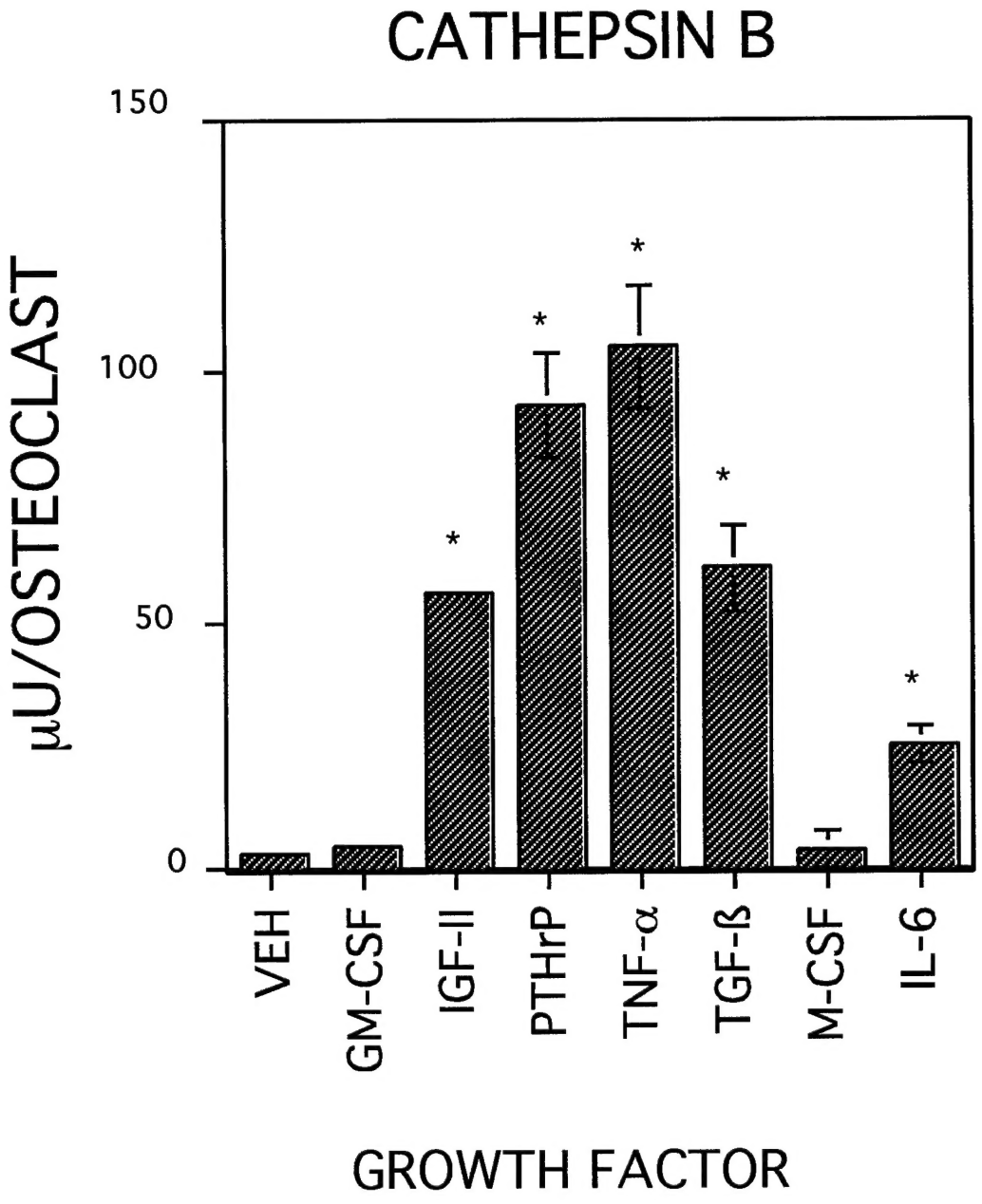
CATHEPSIN B
150
100
50
0
µU/OSTEOCLAST
*
VEH
GM-CSF
IGF-II
PTHrP
TNF-α
TGF-ß
M-CSF
IL-6
GROWTH FACTOR

Figure 5A

B

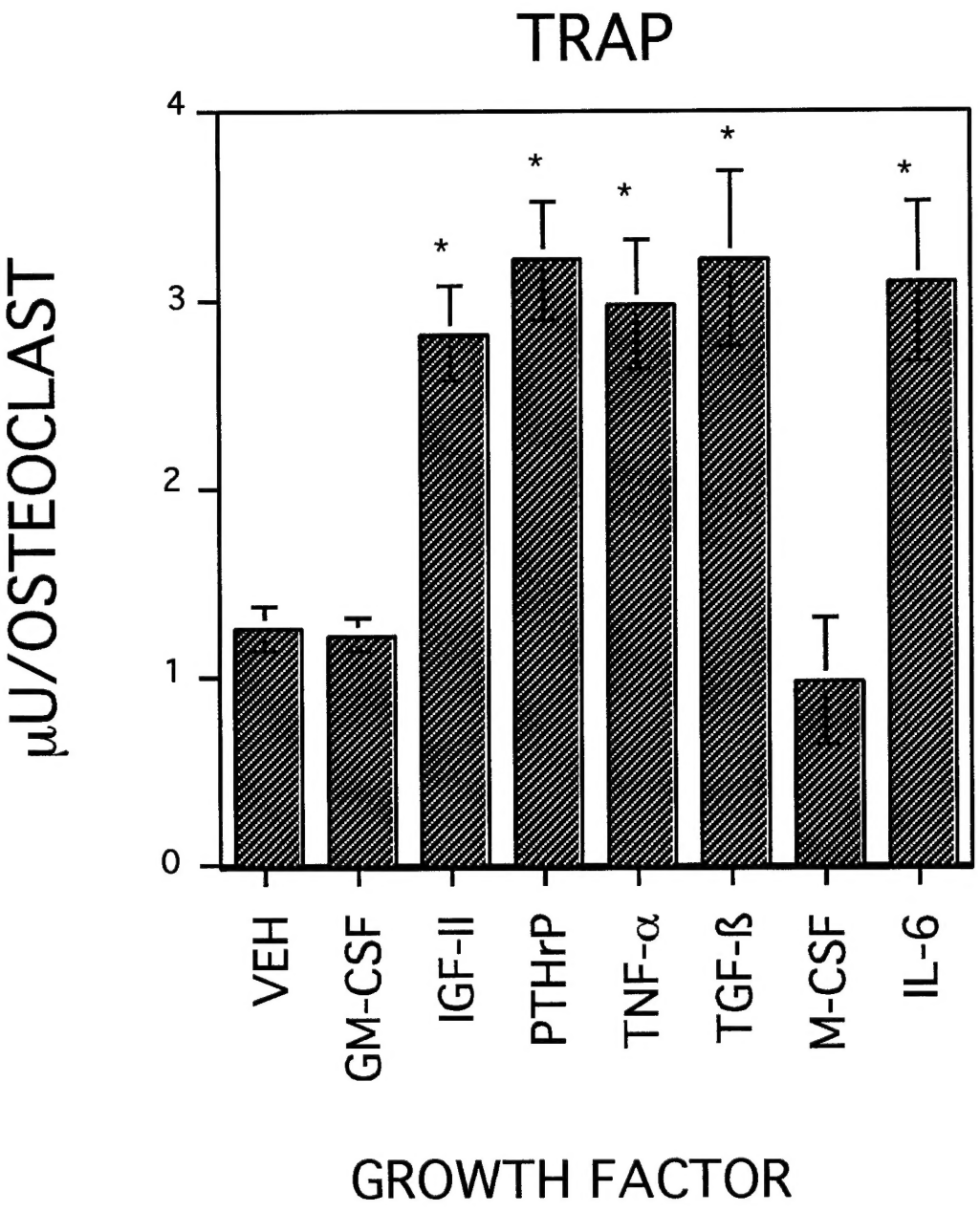
TRAP
µU/OSTEOCLAST
4
3
2
1
0
*
VEH
GM-CSF
IGF-II
PTHrP
TNF-α
TGF-ß
M-CSF
IL-6
GROWTH FACTOR

Figure 5B